

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>A61K 31/00, 31/40</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 95/33454</b> <b>(43) International Publication Date:</b> 14 December 1995 (14.12.95)
<b>(21) International Application Number:</b> PCT/GB95/01301 <b>(22) International Filing Date:</b> 6 June 1995 (06.06.95) <b>(30) Priority Data:</b> 9411363.6 7 June 1994 (07.06.94) GB 9420212.4 6 October 1994 (06.10.94) GB <b>(71) Applicant (for all designated States except US):</b> THE UNIVERSITY OF BIRMINGHAM [GB/GB]; P.O. Box 363, Edgbaston, Birmingham B15 2TT (GB). <b>(71)(72) Applicant and Inventor:</b> AHMED, Asif, Syed [GB/GB]; South Lodge, Bristol Road, Edgbaston, Birmingham B5 7SW (GB). <b>(74) Agents:</b> PEARCE, Anthony, Richmond et al.; Marks & Clerk, Alpha Tower, Suffolk Street Queensway, Birmingham B1 1TT (GB).		<b>(81) Designated States:</b> AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> UTERINE FIBROID TREATMENT  <b>(57) Abstract</b>  The use of a) an angiotensin-converting enzyme (ACE) inhibitor such as Captopril or Enalapril, b) an angiotensin II-receptor antagonist such as Saralasin or Losartan, or c) a renin inhibitor, such as Remikiren or [N-(pyridyl-3-propionyl)-phenylalanyl-histidyl-(3S,4S) ACHPA-isoleucylamino]-2-methyl-2-dihydroxy-1,3-propane, for the manufacture of a medicament for the treatment of uterine fibroids is disclosed. The ACE inhibitor may be used concomitantly or sequentially with a gonadotropin-releasing hormone agonist such as Buserelin or Goserelin.		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

## UTERINE FIBROID TREATMENT

This invention relates to uterine fibroid treatment.

Cellular proliferation and differentiation in uterine tissue is considered to be regulated by ovarian steroids as fibroids appear in the reproductive years and regress after the menopause. Uterine fibroids are most commonly treated by surgery, usually by full or partial hysterectomy, although removal of individual fibroids (myomectomy) is also undertaken at rather greater risk on women who have not completed child bearing. As far as medical treatment is concerned, agonist analogues of LHRH (luteinizing hormone-releasing hormone) such as Buserilin (GnRH analogue) have been employed to suppress oestrogen-progesterone as fibroids are ovarian steroid dependent. Such medical treatments, however, suffer from a variety of side effects such as predisposition to osteopetrosis and are not recommended for long term use.

It is an object of the present invention to provide an alternative medical treatment for uterine fibroids which, it is believed, may have potential for use in situations where the above-mentioned medical treatments are inadvisable.

The present invention is based on the discoveries by the present inventor that angiotensin II (Type II) receptor ( $AT_2$ ) is highly expressed in fibroid tissue compared to normal myometrium (see attached Figs 1(A) to 1(E)), and that angiotensin II stimulates myometrial cell proliferation (see Figs 3(A) and 3(B)).

The present invention, in a first aspect, resides in the use of an angiotensin-converting enzyme (ACE) inhibitor for the manufacture of a medicament for the treatment of uterine fibroids.

The present invention, in a second aspect, resides in the use of an angiotensin II-receptor antagonist for the manufacture of a medicament for the treatment of uterine fibroids.

The present invention, in a third aspect, resides in the use of a renin inhibitor for the manufacture of a medicament for the treatment of uterine fibroids. In this connection, it is to be appreciated that, in the renin-angiotensin system (RAS), renin synthesized by the kidneys and secreted into the circulation cleaves the decapeptide angiotensin I from angiotensinogen. Angiotensin I is converted by ACE to the octapeptide angiotensin II which is the biologically active hormone. Accordingly, the use of a renin inhibitor will also have the effect of inhibiting angiotensin II.

In a fourth aspect, the present invention resides in the use of an angiotensin II receptor ( $AT_1$  and/or  $AT_2$ ) expression inhibitor (including an antisense oligonucleotide directed against the angiotensin II receptor ( $AT_1$  and/or  $AT_2$ ) expression gene) in the manufacture of a medicament for the treatment of fibroids.

With regard to said first aspect of the present invention, namely the use of an ACE inhibitor to suppress angiotensin II expression, it is considered that any ACE inhibitor can potentially be used, including those which are known and which have been used or proposed to be used for ACE inhibition in the treatment of hypertension and congestive heart failure.

For example, ACE inhibitors such as Captopril (very soluble in water) and Enalapril (CAS 75847-73-3) (sparingly soluble in water) have relatively few side effects and are very effective in the treatment of hypertension.

Other ACE inhibitors which may be suitable are:-

Benazepril,

Ramipril,

Lisinopril,

Imidapril 6366A (CAS 89371-44-8) (Arzneim. Forsch./Drug Res., Sept 1992, 42(9), pages 1109-14),

N-[8-amino-1(S)-carboxyoctyl]-L-alanyl-L-proline (AB-47, CAS 120008-53-9)

Pimobendan (CAS 118428-36-7) (Arzneim. Forsch./Drug Res. 43(1) No. 2a, 1993, pages 233 - 235), and

antisense oligonucleotides directed against the ACE gene or against genes for other enzymes in the renin-angiotensin system.

With regard to angiotensin II receptor antagonists, it is considered that suitable antagonists can be found amongst the following:-

Saralasin,

Losartan (DuP753) (CAS 124750-99-8),

PD 123177,

CGP 42112A,

BIBS 39,

BIBS 222,

Sar-Ile-Ang II,

Sar-Thr-Ang II,

Sar-Ala-Ang II,

Sar-Val-Ala-Ang II,

Sar-O-Me-Tyr-Ang II

(see *Arzneim. Forsch./Drug Res.*, 43(1) No. 2a (1993), pages 214 - 246).

As far as renin inhibitors are concerned, it is considered that suitable renin inhibitors may be:-

Remikiren (R<sub>0</sub>42-5892, CAS 126371-83-3)

[N-(pyridyl-3-propionyl)-phenylalanyl-histidyl-(3S,4S) ACHPA-isoleucylamino]-2-methyl-2-dihydroxy-1,3-propane (*Arzneim. Forsch./Drug Res.*, Feb 1993 43(2A), pages 255 - 259).

Other renin inhibitors may be C1-992 (*J. Pharmacol. Exp. Ther.*, 268: 372-9, 1994); non-peptide renin inhibitors containing 2-(((3-phenylpropyl)phosphoryl)oxy)alkanoic acid moieties as P<sub>2</sub>-P<sub>3</sub> replacements (*J. Med. Chem.* 37:486-97, 1994) and antisense oligonucleotides directed against the renin-expression gene.

The ACE inhibitor, angiotensin II-receptor antagonist (e.g. Saralasin, Losartan or PD 123177) or an angiotensin II receptor (AT<sub>1</sub> and/or AT<sub>2</sub>) expression inhibitor (including an antisense oligonucleotide directed against the angiotensin II receptor (AT<sub>1</sub> and/or AT<sub>2</sub>) expression gene) may be used concomitantly or sequentially with a gonadotropin-releasing hormone agonist (GnRH-A) such as Buserelin or Goserelin in the treatment of uterine fibroids to reduce the size thereof.

Accordingly, the present invention further resides in the use of an angiotensin-converting enzyme (ACE) inhibitor or an angiotensin II-receptor antagonist for the manufacture of a medicament for the treatment of uterine fibroids, concomitantly or sequentially with gonadotropin-releasing hormone agonist (GnRH-A) such as Buserelin or Goserelin.

The present invention also resides in the concomitant or sequential use of (a) an ACE inhibitor (e.g. Ramipril, Lisinopril or Enalapril), or an angiotensin II-receptor antagonist (e.g. Saralasin, Losartan or PD 123177) with (b) a gonadotropin-releasing hormone agonist (GnRH-A) such as Buserelin or Goserelin in a method of the treating uterine fibroids.

Such use may comprise administration of a medicament comprising a mixture of (a) and (b), or administration of a medicament containing (a) and a medicament containing (b) either concomitantly or sequentially. Medicament (a) may be administered before medicament (b) or *vice versa*.

The present invention will now be described in further detail.

## **Materials and Methods**

### *Materials*

[<sup>125</sup>I]Angiotensin II (2200 Ci/mmol) was purchased from Amersham International Plc (Amersham, Berks U.K.). Angiotensin II, saralasin and other reagents unless otherwise specified were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). Basic fibroblast growth factor (bFGF) was purchased from RD Systems (Minneapolis, U.S.A.). Losartan (DuP753; (2-n-butyl-4-chloro-5-hydroxymethyl-1-[2-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole) and PD123177 (1-(4-amino-3-methylphenyl)-methyl-5-diphenyl-acetyl-4,5,6,7-tetrahydro-1H-imidazo [4, 5-C] pyridine-6-carboxylic acid) were from DuPont Merck Pharmaceuticals and kindly provided by Dr. N.M. Barnes, University of Birmingham. Components for the culture medium were obtained from Flow Laboratories (High Wycombe, Berks., U.K.).

*Source of tissue*

Myometrium was collected from women undergoing total abdominal hysterectomy. Patients selected had no apparent endocrinological problems and no local organic pathologies. All tissues were obtained from 20 - 48 year old women with a history of regular menstrual cycles. None of the patients had taken any hormonal medication for at least 3 months before the surgery. The tissue was rinsed in sterile saline and rapidly frozen over dry-ice, wrapped in Parafilm to prevent dehydration and stored at -70°C until they were sectioned for receptor autoradiographic studies.

*Preparation of tissue sections*

Frozen myometrium and fibroid tissues were surrounded in embedding medium (OCT compound, Miles Scientific) before 20  $\mu$ m sections were cut using a cryostat (-15 to -19°C) and thaw mounted onto gelatin-coated glass slides for receptor autoradiographic studies. Sections were stored (less than 2 week) desiccated at -80°C until used.

*Myometrial cell preparation*

Tissue samples were placed into sterile calcium and magnesium-free HBSS containing penicillin (100 U/ml) and streptomycin (100  $\mu$ g/ml) for transfer to the laboratory. Myometrial cells were isolated by collagenase digestion. Briefly, myometrium was finely chopped and incubated in HBSS containing 1mg/ml collagenase IA, 20mM Hepes, 100 U/L penicillin, 100  $\mu$ g/ml streptomycin and 5  $\mu$ g/ml fungizone at 37°C until the cells were dispersed. Myometrial cells were obtained by centrifugation for 15 minutes at 400g over 60% Percoll and the cells



washed with HBSS. Myometrial cells were characterised by their ability to respond to oxytocin and staining for actin.

*[<sup>125</sup>I]Angiotensin II autoradiography*

Slide mounted human myometrial/fibroid sections were removed from storage and allowed approximately 30 min to equilibrate to room temperature. To reduce endogenous levels of angiotensin II in the tissue, the sections were preincubated for 60 min in incubation buffer (mM; sodium chloride, 150; sodium dihydrogen phosphate, 50; magnesium chloride, 10; ethyleneglycol-bis-( $\beta$ -amino-ethyl ether)-N,N'-tetra-acetic acid, 5 and 0.4% w/v bovine serum albumin, pH 7.4) at 25°C. The slides were then incubated in incubation buffer which contained 0.1 nM [<sup>125</sup>I]angiotensin II in the absence (total binding) or presence of competing compound (either 1.0  $\mu$ M unlabelled angiotensin II, 1.0  $\mu$ M DuP753, 1.0  $\mu$ M PD123177 or 1.0  $\mu$ M DuP753 plus 1.0  $\mu$ M PD123177) for 60 min at 25°C. Immediately following incubation, the tissue sections were washed in ice-cold incubation buffer for 2 min and dipped (1 s) in ice-cold distilled water to remove buffer salts. The sections were rapidly dried in a stream of cold dry air and exposed to Hyperfilm-[<sup>3</sup>H] (Amersham International) in X-ray cassettes together with [<sup>125</sup>I] standards (Amersham International) for 10 days. Autoradiographic films were developed in Kodak LX 24 developer (5 min) and Kodak Unifix (5 min) and were quantitated by reference to [<sup>125</sup>I] standards (fmol/mg tissue; Amersham) using image analysis.

*Cell culture and cell proliferation studies*

Cells were maintained in 175 cm<sup>2</sup> flasks in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal calf serum (FCS), 1% L-glutamine, 20 mM Hepes and 1% antibiotic-antimycotic mixture (10,000

U penicillin, 10  $\mu\text{g}$  streptomycin, and 25  $\mu\text{g ml}^{-1}$  fungizone) at 37°C in a humidified atmosphere of air/CO<sub>2</sub>(19:1) until 95% confluent. Thereafter, the cells were trypsinised, washed and re-plated at a density of  $10 \times 10^3$  cells per well in 24-well plates in 1 ml of DMEM with 10% FCS for the proliferation studies. After 24 hrs, the medium was removed and replaced with serum-free DMEM. After a further 24 hrs, the experiments were initiated on quiescent monolayers of myometrial cells. To the serum-starved myometrial cells was added either angiotensin II (0.1 $\mu\text{M}$  or 1.0 $\mu\text{M}$ ) in the presence or absence of 1% FCS or bFGF (20 ng/ml) or FCS (1%) alone or DuP753 with or without angiotensin II (0.1  $\mu\text{M}$ ) and cell growth measured at day 4 and day 6 by counting cell numbers in a Coulter counter as described below. The corresponding medium was replaced every 24 hrs with fresh medium.

#### **Calibration of the Coulter Counter**

Calibration of the Coulter counter was achieved by counting the myometrium cell suspension at reduced aperture size ranging from 0 to 32. A calibration curve was constructed from the cell counts on the function of the aperture size, and the aperture size corresponding to the middle point of the plateau part of the calibration curve was used for the cell counting. The aperture size used for myometrium cell counting was 16.

#### **Counting of the Myometrium Cells**

At defined time-points, the growth media was removed and the cells were washed with 1 ml of isotonic saline (0.9% sodium chloride), followed by the addition of 1 ml of 10 mM HEPES buffer containing 1.5 mM magnesium chloride, and 2 drops of Zap-oglobin (Coulter Electronics Ltd., Luton, Beds., UK) containing 2.5% acetic acid to each

well. The 24-well plate was allowed to incubate at 37°C for 10 min. After which, the suspended cells were pipetted up and down to disperse the cell clusters. The cell suspension was then added to 9 ml of counting solution containing 0.9% sodium chloride and 0.5% formalin in a Coulter counter container. Each well was washed three times with the counting solution from the same container and effluent collected in the same container. The number of cells in each well was counted at the aperture size of 16 on the Coulter counter (Coulter Electronics Ltd., Luton, Beds U.K.). Three readings were taken for each well and a mean value of the three readings obtained. The mean ( $\pm$  SD) value of cell counts from the triplicate wells was calculated.

## Results and Discussion

### *Expression of angiotensin II receptor subtypes*

With the development of non-peptide angiotensin II receptor (AT) antagonists, attention has focused on the AT receptor as a target to pharmacologically manipulate the renin-angiotensin system. Using quantitative receptor autoradiography, we have pharmacologically characterised the presence of AT<sub>2</sub> receptors on non-pregnant myometrium and fibroids (Figure 1). Dark regions indicate high density of labelled receptors. It is clear from the Figure 1 that the fibroid region contains a higher density of angiotensin II receptors than normal myometrium. Figure 1(A) to 1(E) are autoradiograms of the distribution of [<sup>125</sup>I]angiotensin II binding to adjacent sections (20  $\mu$ m) of the human non-pregnant myometrium containing a fibroid. In Figure 1(A), total binding of such labelled angiotensin II is illustrated. Figure 1(B) shows binding in the presence of the AT<sub>1</sub> receptor selective ligand DuP753 (1.0  $\mu$ m). Figure 1(C) shows binding in the presence of the AT<sub>2</sub> receptor selective ligand PD123177 (1.0  $\mu$ m). Figure 1(D) shows binding in the

presence of DuP753 (1.0  $\mu$ M) plus PD123177 (1.0  $\mu$ M). Figure 1(E) shows binding in the presence of unlabelled angiotensin II (0.1 nM). Dark regions indicate high densities of labelled receptors. The difference in density between Figures 1(A) and 1(B) represents AT<sub>1</sub> receptor specific binding. This receptor subtype is weakly expressed in myometrium. The difference in density between Figures 1(A) and 1(C) represents AT<sub>2</sub> receptor specific binding. The fibroid region in Figure 1(C) shows a lower AT receptor expression than the same region in Figure 1(B), thus indicating that the fibroid has predominantly AT<sub>2</sub> type receptors. The fibroid had a higher density of angiotensin II receptors than normal myometrium. The difference between Figure 1(A) and 1(D) represents specific AT<sub>1</sub> plus AT<sub>2</sub> receptor density, and the difference between Figure 1(D) and 1(E) represents a newly discovered, non-AT<sub>1</sub>/non-AT<sub>2</sub> angiotensin II recognition site. The intensity of expression of such non-AT<sub>1</sub>/non-AT<sub>2</sub> angiotensin II recognition site in the fibroid tissue only disappears in the presence of cold angiotensin II (1 $\mu$ M), thus indicating that this is an additional high affinity angiotensin II recognition site. This newly discovered site is pharmacologically distinct from known AT receptor subtypes, and Sar-Thr-angiotensin II and Sar-Ala-angiotensin II can selectively displace [<sup>125</sup>I]angiotensin II from this site (Fig 2).

#### *Angiotensin II-induced myometrial cell proliferation*

The results of growth experiments are given in attached Figure 3(A) and 3(B) which are graphs plotting mean cell count against time in days. It will be seen from Figure 3(A) that the presence of angiotensin II (0.1  $\mu$ M) in culture medium has a significant effect on myometrial cell growth and that after six days in culture there is approximately a 100% increase in myometrial cell proliferation compared to cells in culture medium devoid of angiotensin II. It is also interesting to note that the angiotensin

II-induced response is comparable to that induced by a classic growth factor such as bFGF (Table 1).

Table 1

Days	0 day (+/-SD)	4 days (+/-SD)	6 days (+/-SD)
Control (1% FCS)	65.83 +/-1.52	127.00 +/-12.53	146.33 +/-16.22
Ag II (0.1 $\mu$ M in 1% FCS)	64.33 +/-2.73	167.33 +/-21.55	333.67 +/-7.51
bFGF (20 ng/ml in 1% FCS)	63.90 +/-1.88	167.33 +/-25.11	307.33 +/-49.69

In addition, angiotensin II receptor antagonists blocked the effect of angiotensin II on cell proliferation. It can be seen in Figure 3(B) as an example that DuP753 inhibits the effects of angiotensin II on cell proliferation. Preliminary data also shows that PD177123 also inhibits angiotensin II-mediated cell growth. Consequently, it is reasonable to infer that reduction of the level of angiotensin II or blocking of the high affinity angiotensin II binding sites in the fibroid will lead to a reduction in, or inhibition of, fibroid growth.

#### *Patient treatment regimes*

The invention proposes to treat patients in one of the following ways:-

- (1) By oral administration of the drug and this may be the preferred method of treatment.
- (2) intravaginal administration using a vaginal ring
- (3) adding drug via a intrauterine device
- (4) directly at the site of the tumour using an ultrasound directed local injection into the fibroid (as some of these compounds are water soluble, they could be placed in an oil-based solution and then injected so as to give slow release of the active drug).
- (5) as a pellet inserted directly at the site of the tumour using the long-acting near-zero order releasing system with the aid of laproscopy.

*In the accompany drawings*

Figure 1. Autoradiograms of the distribution of [ $^{125}$ I]angiotensin II binding to adjacent sections (20  $\mu$ m) of the human non-pregnant myometrium containing a fibroid.

(A), total binding;

(B), binding in the presence of the AT<sub>1</sub> receptor selective ligand DuP753 (1.0  $\mu$ M);

(C), binding in the presence of the AT<sub>2</sub> receptor selective ligand PD123177 (1.0  $\mu$ M);

(D), binding in the presence of DuP753 (1.0  $\mu$ M) plus PD123177 (1.0  $\mu$ M);

(E), binding in the presence of unlabelled angiotensin II (0.1nM).

Dark regions indicate high densities of labelled receptors. The difference in density between (A) and (B) represents AT<sub>1</sub> receptor specific binding (this receptor subtype is not detected in myometrium); difference in density between (A) and (C) represents AT<sub>2</sub> receptor specific binding; difference between (A) and (D) represents specific AT<sub>1</sub> plus AT<sub>2</sub> receptor density and the difference between (D) and (E) represents the newly discovered, non-AT<sub>1</sub>/non AT<sub>2</sub> angiotensin II recognition site. Note the intensity of expression of such non-AT<sub>1</sub>/non-AT<sub>2</sub> angiotensin II recognition site in the fibroid tissue which only disappears in the presence of cold angiotensin II suggesting that the fibroid expresses high levels of the binding site.

Figure 2. Autoradiograms of the distribution of [ $^{125}$ I]angiotensin II binding to adjacent sections (20  $\mu$ m) of the human non-pregnant myometrium (Myo) and fibroid (Fib).

(A), total binding;

- (B), binding in the presence of the AT<sub>1</sub> receptor selective ligand DuP753 (1.0  $\mu$ M);
- (C), binding in the presence of the AT<sub>2</sub> receptor selective ligand PD123177 (1.0  $\mu$ M);
- (D), binding in the presence of DuP753 (1.0  $\mu$ M) plus PD123177 (1.0  $\mu$ M);
- (E), binding in the presence of unlabelled angiotensin II (AngII, 0.1 nM);
- (F), binding in the presence of unlabelled angiotensin I (AngI, 0.1 nM);
- (G), binding in the presence of unlabelled angiotensin III (AngIII, 0.1 nM);
- (H), binding in the presence of unlabelled Sar-Thr-angiotensin II (0.1 nM);
- (I), binding in the presence of unlabelled Amino-Phe-angiotensin II (0.1 nM);
- (J), binding in the presence of unlabelled Sar-Ala-angiotensin II (0.1 nM).

Dark regions indicate high densities of labelled receptors.

Figure 3(A) Effect of angiotensin II on myometrial cell proliferation. Myometrium cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) FCS, containing 1% L-glutamine, 20 mM HEPES and 1% antibiotic-antimycotic mixture (10,000 U penicillin, 10 mg streptomycin) at 37°C in a humidified atmosphere of air/CO<sub>2</sub> (19:1) until 95% confluent. For the proliferation studies, the cells were plated at a density of  $10 \times 10^3$  cells per well in 24 well plates in 1 ml of DMEM with 10% FCS. After 24 hrs the medium was removed and replaced with serum-free DMEM. After a further 24 hrs, the experiment was initiated on quiescent monolayers of myometrial cells. To each of the wells in triplicate was added 1 ml of angiotensin II (0.1  $\mu$ M) or DMEM alone, in the presence or absence of 1% FCS. The cell growth

was measured at day 4 and day 6 by counting cell numbers in a Coulter Counter. Each point represents the mean  $\pm$  sem of triplicate determinations from a single experiment, typical of three others.

Figure 3. (B) Effect of AT<sub>1</sub> receptor ligand (DuP753) on angiotensin II-induced myometrial cell proliferation.

Myometrium cells were maintained in Dulbecco's modified Eagle's medium (MD) with 10% (v/v) FCS, containing 1% L-glutamine, 20 mM HEPES and 1% antibiotic-antimycotic mixture (10,000 U penicillin, 10 mg streptomycin) at 37°C in a humidified atmosphere of air/CO<sub>2</sub> (19:1) until 95% confluent. For the proliferation studies, the cells were plated at a density of  $10 \times 10^3$  cells per well in 24 well plates in 1 ml of DMEM with 10% FCS. After 24 hrs the medium was removed and replaced with serum-free DMEM. After a further 24 hrs, the experiment was initiated on quiescent monolayers of myometrial cells. To each of the wells, in triplicate, was added 1 ml of 1% FCS as control or angiotensin II (0.1  $\mu$ M), or DuP753 (1  $\mu$ M) or DuP753 plus angiotensin II (0.1  $\mu$ M) in the presence of 1% FCS. The cell growth was measured at day 4 and day 6 by counting cell numbers in a Coulter Counter. Each point represents the mean  $\pm$  sem of triplicate determinations from a single experiment, typical of three others.

A further series of experiments were conducted as described below:-

#### *Immunocytochemistry*

Serial 3  $\mu$ m sections of formalin-fixed, paraffin-embedded tissue were used for immunohistochemistry. Sections were de-paraffinized by incubation for 5 min with HistoClear and hydrated through methanol to water. Endogenous peroxidase activity was quenched by 0.3% (v/v)



hydrogen peroxide in methanol for 10 minutes. The primary antibodies were rabbit polyclonal antibodies raised against the human renin, angiotensin converting enzyme, ANG1, ANG II and AT<sub>1</sub> receptor. Non-immune goat serum (10% in 0.01 mol/l PBS, pH 7.2) was used as a dilution of the primary antibody to reduce non-specific binding.

Amplification of the primary antibody reaction was achieved using a goat anti-rabbit secondary antibody (diluted 1:200 in 0.01 mol/l PBS, pH 7.2) for 30 min followed by a complex of streptavidin (Dako Ltd, Bucks, UK) and biotinylated peroxidase (Dako Ltd, Bucks, UK). Finally, the binding was visualised by the addition of 0.5 mg/ml diaminobenzidine (Sigma Chemical Co. Ltd, Poole, Dorset, UK) and 0.01% hydrogen peroxide in 0.01 mmol/l PBS to the antigen-antibody complex. Between each step the sections were washed in 3 x 200 ml of 0.1% (v/v) polyoxyethylene-10-oleoyl-ether in 0.01 mmol/l PBS, pH 7.2, over a period of 15 min. All incubations of antisera were carried out at room temperature in a wet chamber mounted on a rocking tray which ensures a movement of antiserum over the whole section. The sections were counterstained with Mayers Haematoxylin, dehydrated and mounted.

### *Results*

Using the immunocytochemistry described above, the primary antibodies raised against renin, angiotensin converting enzyme, angiotensin I and angiotensin II were used to show that the components of the renin-angiotensin system (RAS) are expressed in myometrium and fibroids. This suggests that there is local RAS within the tissue itself, further supporting the functional studies. In addition, immunoreactive AT<sub>1</sub> receptor protein was localised in myometrial smooth muscle cells and around the blood vessels, indicating that myometrium and fibroid tissues contain AT<sub>1</sub> receptors in addition to AT<sub>2</sub> receptors which were clearly

identified using autoradiography results. Evidence also indicates that there is intense vascularization around the uterine fibroid and also within the fibroids as QBN10 (a marker of endothelial cells) intense endothelial cell staining in immunohistochemical sections.

*Cell culture and measurement of [ $^3\text{H}$ ]-thymidine incorporation*

Myometrial cells prepared as described previously were resuspended in culture medium containing DMEM supplemented with 10% (v/v) FCS, 10 mM L-glutamine, 20 mM hepes, 100 U/ml penicillin and 100  $\mu\text{g/ml}$  streptomycin in 24 well plates at a density of approximately  $250 \times 10^3$  cells per well and maintained at 37°C in a humidified atmosphere of air/CO<sub>2</sub> (19:1).

DNA synthesis was assayed by measuring [ $^3\text{H}$ ]-thymidine incorporation into DNA. Subconfluent myometrial cells were made quiescent by incubation with serum-free medium for 48h, the medium being replaced with serum-free medium containing 0.2% (w/v) bovine serum albumin (BSA) and [ $^3\text{H}$ ]-thymidine (0.2  $\mu\text{Ci/well}$ ). The cells were further incubated for 48 h in the presence of angiotensin-converting enzyme (ACE) inhibitor (0.1 and 1.0 mM Captopril, hatched and solid columns respectively) or angiotensin II (100 nM AgII, crossed column). The medium was then removed and the cells incubated with 10% cold trichloroacetic acid (TCA) at 4°C for 15 min. TCA-precipitated material was redissolved in 0.2 M NaOH and the radioactivity determined by liquid scintillation counting in a  $\beta$ -scintillation analyser. Positive and negative controls for proliferation were established by incubating cells with 10% fetal calf serum (square column) and by growth in the absence of the agonist in serum-free medium (open column).

### *Results*

As shown in Fig 4A, DNA synthesis was reduced by almost 50% of control (open column- 0% FCS) in the presence of ACE inhibitor. This suggests that myometrial cells have the capacity to generate endogenous angiotensin II which promotes myometrial cell growth. Use of ACE inhibitors will reduce the ability of these cells to proliferate.

In another series of experiments, using the procedures described above in relation to Figs. 3A and 3B, the results as shown in Fig. 4B were obtained. As Fig. 4B shows, angiotensin II-mediated cell proliferation is significantly inhibited when the cells are incubated with 1.00  $\mu$ M of DuP753 or PD123177 or/and DuP753 plus PD123177. This suggests that in myometrial cells, angiotensin II stimulates cells proliferation via AT<sub>1</sub> or AT<sub>2</sub> receptor subtype.

Method of treatment and drug administration:

Example

Treatment with angiotensin converting enzyme inhibitor Ramipril.

Start patient on 2.5 mg once daily for one week. Increase dose to 5 mg once daily for 12 weeks then increase dose to 10 mg for a further 12 weeks. Monitor size of fibroid and continue at this dose until fibroids fibroid/uterus reduces in size by 50%. Reduce dose to 5 mg once daily for as long as required. Drug may be orally administered.

## CLAIMS

1. The use of an angiotensin-converting enzyme (ACE) inhibitor for the manufacture of a medicament for the treatment of uterine fibroids.
2. The use as claimed in claim 1, wherein the ACE inhibitor is selected from Captopril, Enalapril, Benazepril, Ramipril, Lisinopril, Imidapril, 6366A, N-[8-amino-1(S)-carboxyoctyl]-L-alanyl-L-proline and Pimobendan.
3. The use of an angiotensin II-receptor antagonist for the manufacture of a medicament for the treatment of uterine fibroids.
4. The use as claimed in claim 3, wherein the angiotensin II receptor antagonist is selected from Saralasin, Losartan, PD 123177, CGP 42112A, BIBS 39, BIBS 222, Sar-Ile-Ang II, Sar-Thr-Ang II, Sar-Ala-Ang II, Sar-Val-Ala-Ang II and Sar-O-Me-Tyr-Ang II.
5. The use of a renin inhibitor for the manufacture of a medicament for the treatment of uterine fibroids.
6. The use as claimed in claim 6, wherein the renin inhibitor is selected from Remikiren, [N-(pyridyl-3-propionyl)-phenylalanyl-histidyl-(3S,4S) ACHPA-isoleucylamino]-2-methyl-2-dihydroxy-1,3-propane, C1-992, and non-peptide renin inhibitors containing 2-(((3-phenylpropyl)phosphoryl)oxy)alkanoic acid moieties as P<sub>2</sub>-P<sub>3</sub> replacements.

7. The use of an angiotensin II receptor expression inhibitor in the manufacture of a medicament for the treatment of fibroids.
8. The use as claimed in claim 1, 2 or 7, concomitantly or sequentially with a gonadotropin-releasing hormone agonist.
9. The use as claimed in claim 8, wherein the gonadotropin-releasing hormone agonist is Buserelin or Goserelin.
10. The use of an angiotensin-converting enzyme (ACE) inhibitor, an angiotensin II-receptor antagonist or an angiotensin II receptor expression inhibitor for the manufacture of a medicament for the treatment of uterine fibroids, concomitantly or sequentially with gonadotropin-releasing hormone agonist.
11. The concomitant or sequential use of (a) an ACE inhibitor, an angiotensin II-receptor antagonist or an angiotensin II receptor expression inhibitor with (b) a gonadotropin-releasing hormone agonist (GnRH-A) in a method of the treating uterine fibroids.

1/3

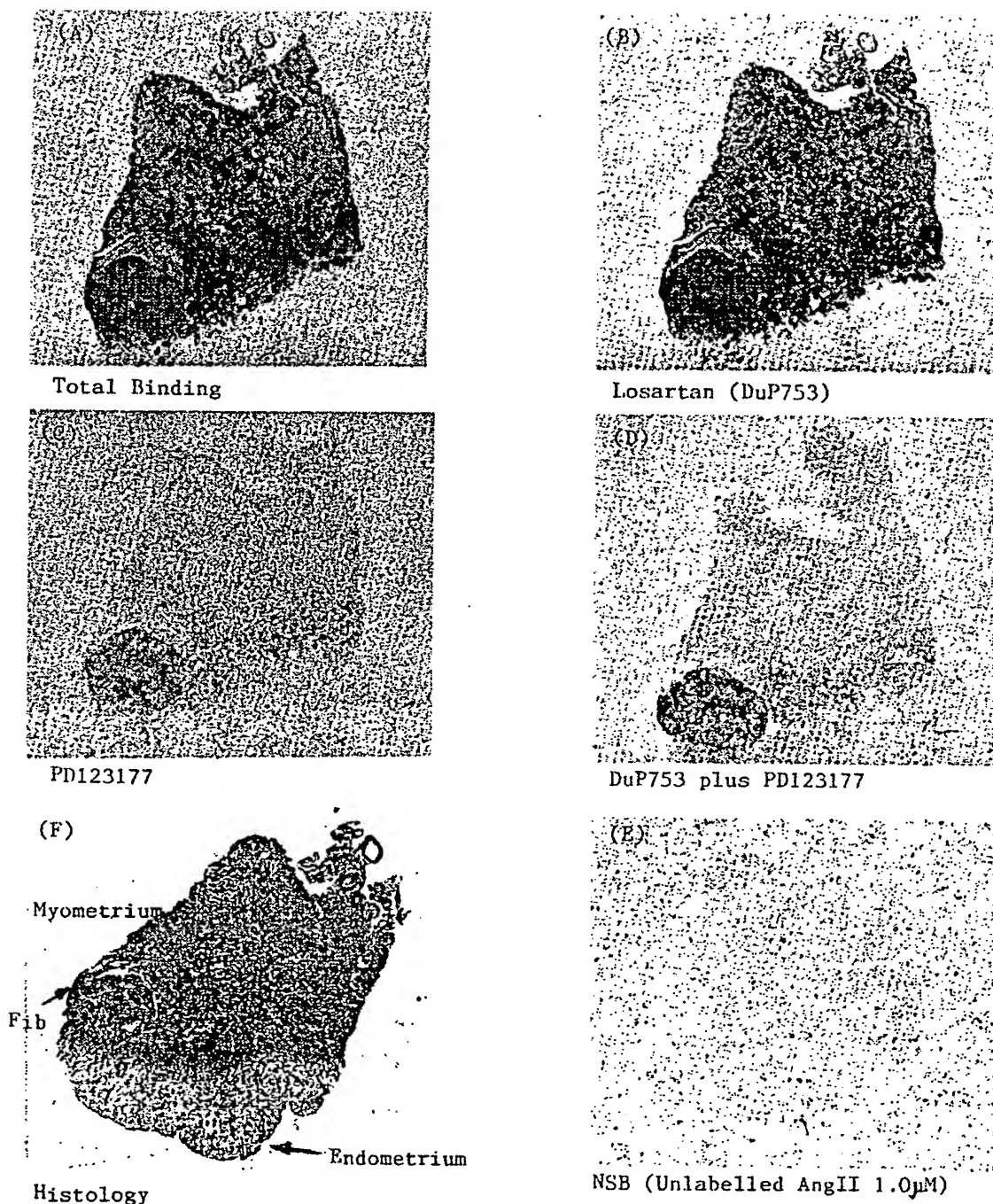
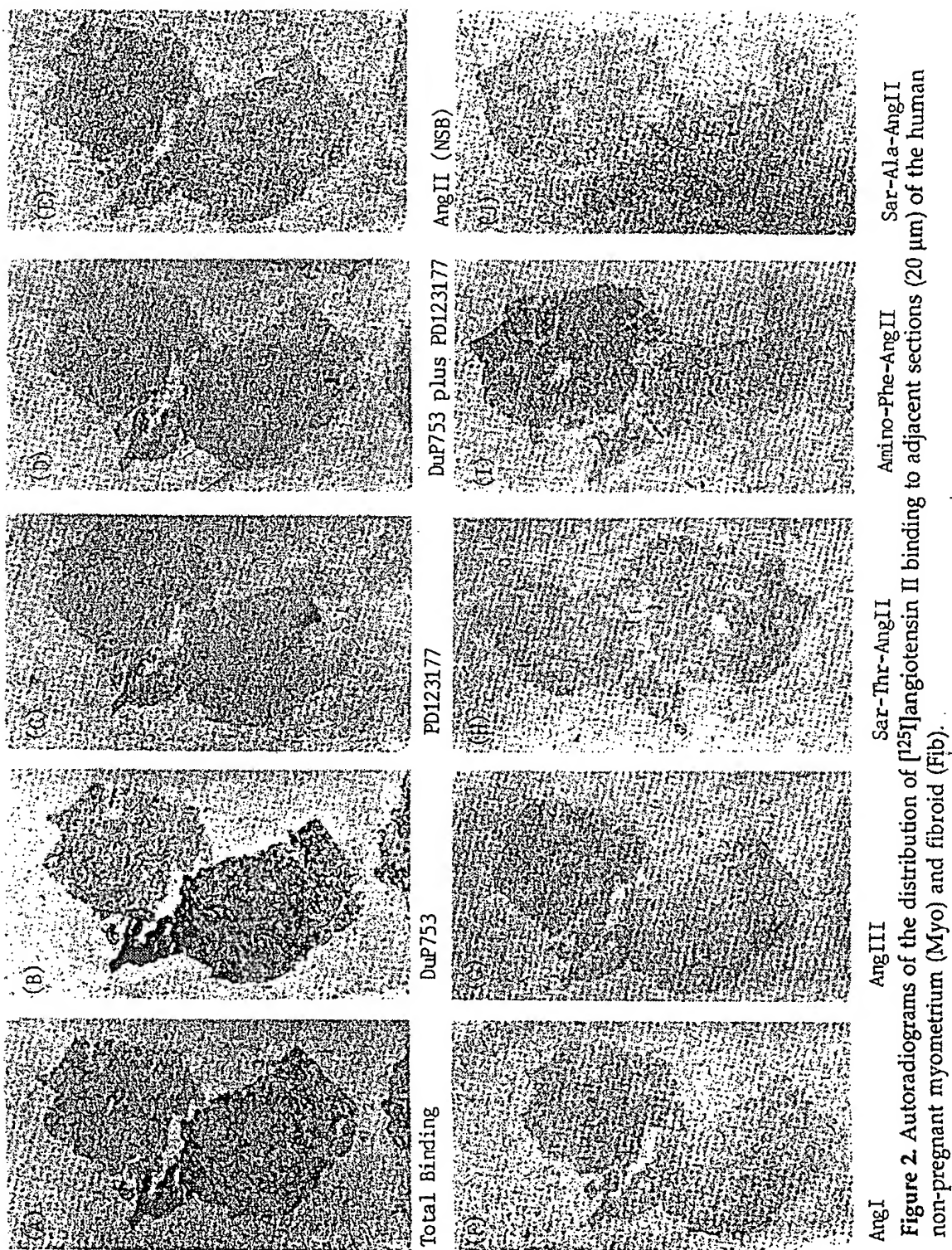


Figure 1. Autoradiograms of the distribution of  $[^{25}\text{I}]$  angiotensin II binding to adjacent sections (20  $\mu\text{m}$ ) of the human non-pregnant myometrium containing a fibroid.

2 / 5



3 / 5

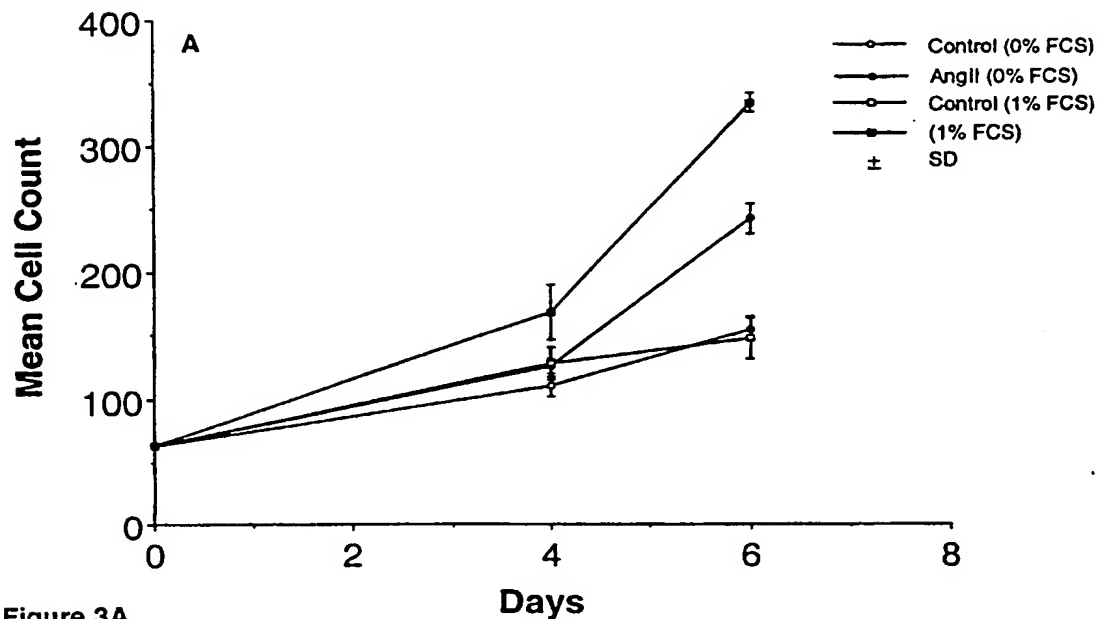
**Effect of angiotensin II on myometrial cell growth**

Figure 3A

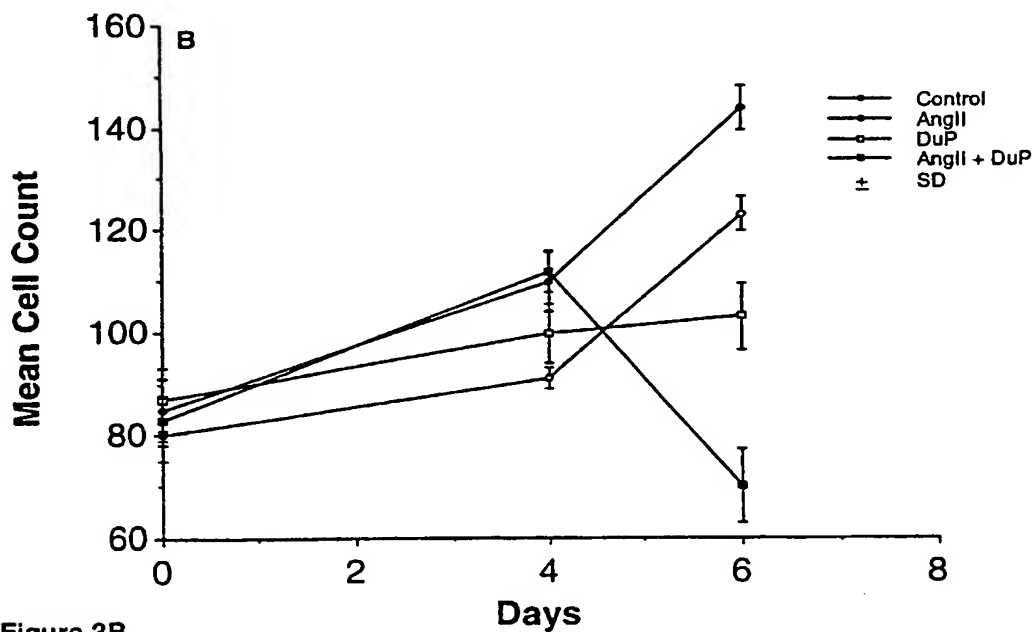
**Effect of DuP753 on angiotensin II on myometrial cell growth**

Figure 3B



4 / 5

# Effect of ACE inhibitor on cell proliferation

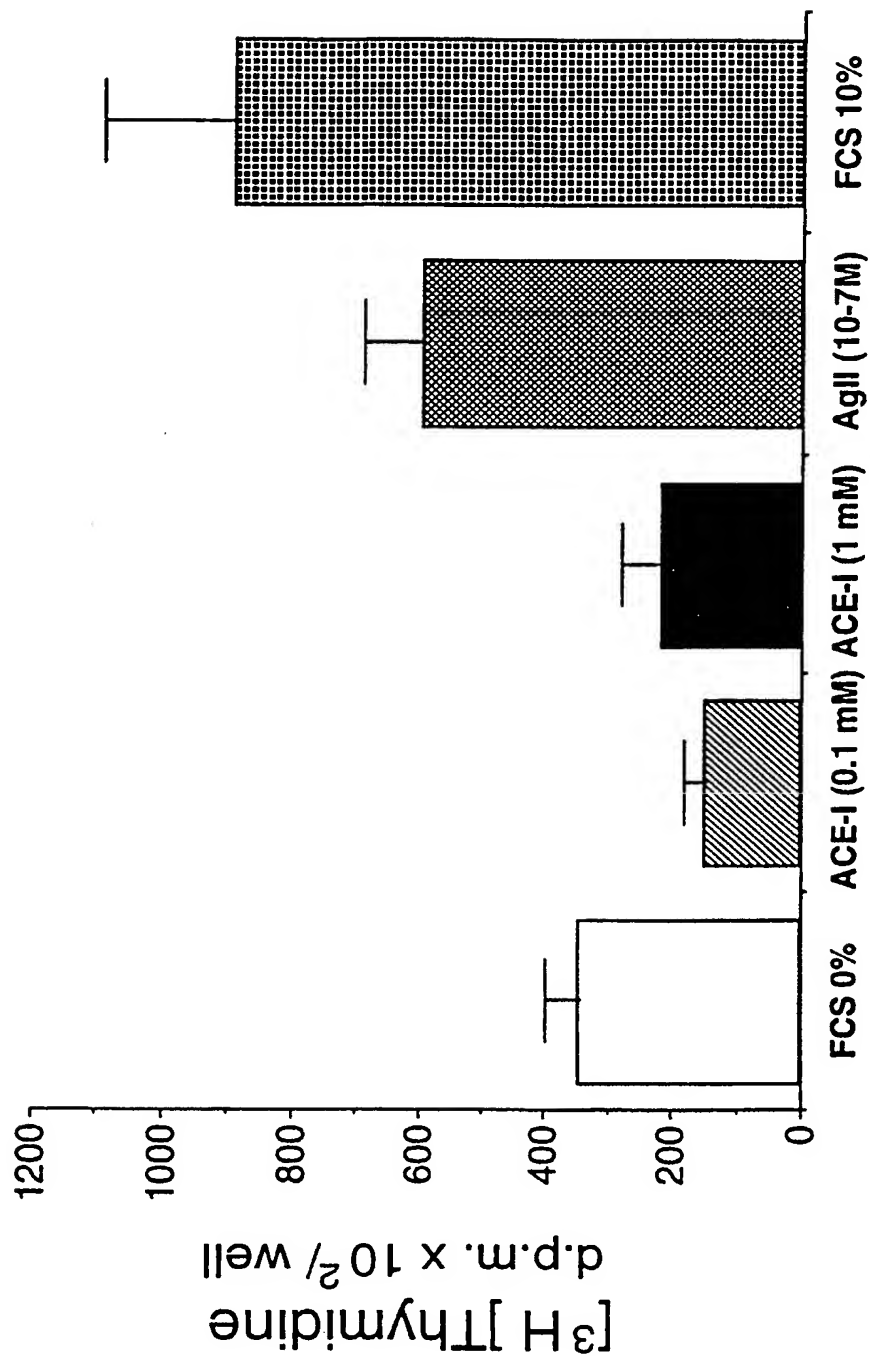
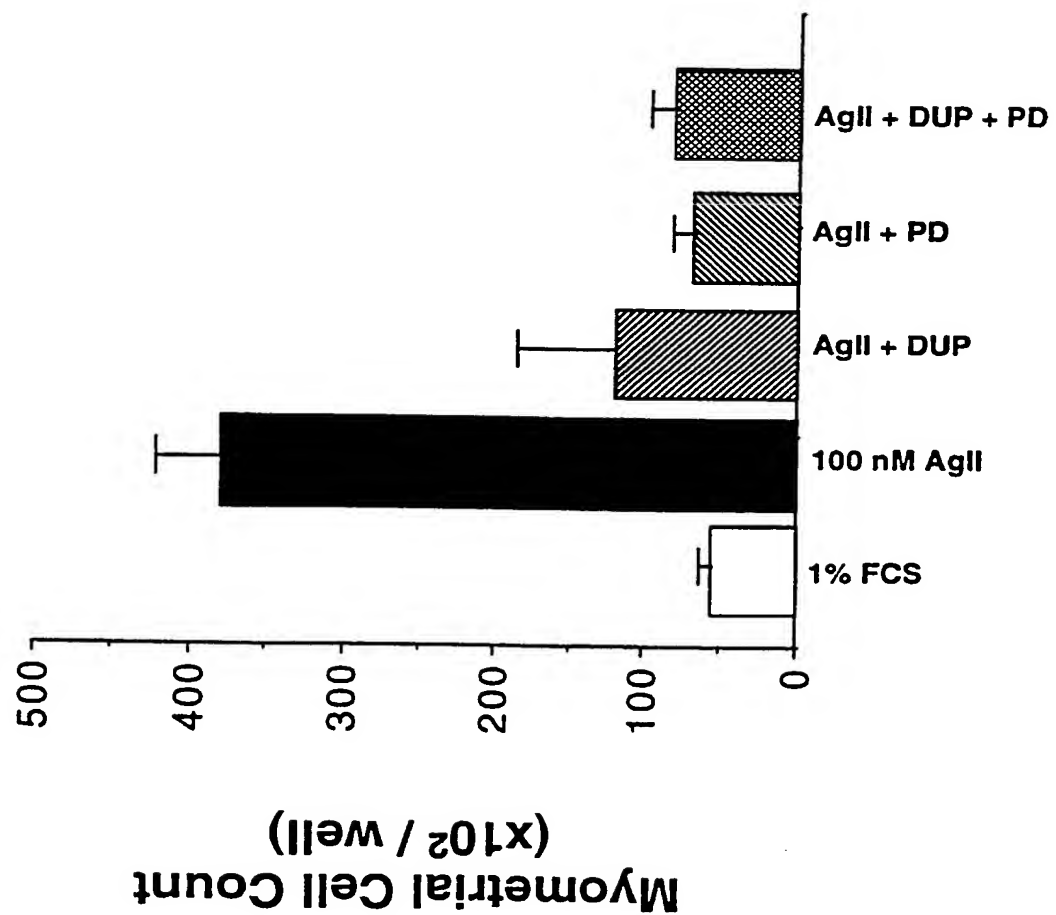


Fig 7A

Treatment Regimes

6/5

**Effect of AT receptor antagonists on angiotensin II-mediated cell growth**

## INTERNATIONAL SEARCH REPORT

Internat Application No  
PCT/GB 95/01301A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 A61K31/00 A61K31/40

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	EUROPEAN HEART JOURNAL, vol. 16, Suppl. C, 1995 UNITED KINGDOM, pages 33-37, NAGASAWA K. ET AL 'EXTRACELLULAR MATRIX DEPOSITION IN HYPERTENSIVE HEARTS ANTIFIBROTIC EFFECTS OF RAMIPRIL' See abstract	7
P,Y	- " -	1-11
Y	--- EXPERIMENTAL AND MOLECULAR PATHOLOGY, vol. 35, no. 2, 1981 USA, pages 265-276, CAMPBELL-BOSWELL M. ET AL 'EFFECTS OF ANGIOTENSIN II AND VASOPRESSIN ON HUMAN SMOOTH MUSCLE CELLS IN VITRO' see page 274, line 16-20 --- -/--	1-11

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

## \* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*&\* document member of the same patent family

Date of the actual completion of the international search

4 October 1995

Date of mailing of the international search report

27. 10. 95

Name and mailing address of the ISA  
European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,  
Fax (+ 31-70) 340-3016

Authorized officer

Herrera, S

## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/GB 95/01301

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,Y	US-A-5 407 955 (BRYANT HENRY U ET AL) 18 April 1995 see column 1, line 9-14 see column 8, line 9-43 ----	1-11
Y	POSTGRADUATE MEDICINE, vol. 94, no. 3, 1993 USA, pages 129-134, CHRISTIANSEN JK 'THE FACTS ABOUT FIBROIDS. PRESENTATION AND LATEST MANAGEMENT OPTIONS.' see page 130, left column, line 12-19 see page 131, right column, line 23 - page 132, right column, line 35 ----	1-11
A	AMERICAN JOURNAL OF PHYSIOLOGY : HEART AND CIRCULATORY PHYSIOLOGY, vol. 31, no. 5, 1992 USA, pages R754-R760, LUMBERS E R ET AL ' ACUTE EFFECTS OF CAPTOPRIL AN ANGIOTENSIN-CONVERTIN ENZYME INHIBITOR ON THE PREGNANT EWE ANT FETUS' see the whole document -----	1-11

## Information on patent family members

PCT/GB 95/01301

Form PCT/ISA/210 (patent family annex) (July 1992)

This Page Blank (uspto)